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Characterization Of Lactic Acid Bacteria (LAB) Origin Sumatran Orangutan (*Pongo abelii*) In Zoo Bukittinggi Based On Analysis 16 S rRNA

Siti Hajar[1], Safika[2], Darmawi[2], Wenny Novita Sari[2], Erdiansyah Rahmi[3], Yandi Syahputra[3], Nurliana[3], Rinidar[3]

[1]Post graduate, Public Health Veterinary, Syah Kuala University
[2]Microbiology Laboratory Veteriney Medicine Syiah Kuala University
[3]Medical Veterinary Syah Kuala University
Email for correspondence: safika@unsyiah.ac.id

Abstract

The purpose of this study was to characterize the bacterial 16S rRNA gene of Sumatran orangutan (*Pongo abelii*) Bukittinggi West Sumatera zoo. The sample used in this study are lactic acid bacteria of the Sumatran orangutan (*Pongo abelii*) derived from zoo Bukittinggi West Sumatra Indonesia. This study was an exploratory study that conducted at the Laboratory through several stages. The first stage was the isolation of LAB from faeces of Sumatran orangutans using MRS agar medium and then cultured in a liquid medium NB. The next stage was the isolation of total DNA, and then, the third stage was the amplification of the 16S rRNA gene and agarose gel electrophoresis. Then,in the fourth stage of determining the DNA sequence and analysis of DNA sequence homology. The final stage was the computational analysis of 16S rRNA gene Sumatran orangutan (*Pongo abelii*). The results showed that lactic acid bacteria (LAB) from the Sumatran orangutan (*Pongo abelii*) is close to lactic acid bacteria *Lactobacillus helveticus* strain IMAU50151 with the levelof similarity of 89%. It is possible that these bacteria is a new species or the species that LAB has not been reported in Genbank.

Key words : Lactic acid bacteria (LAB), Sumatran Orangutan (Pongo abelii), Lactobacillu-helveticus

Background

Lactic acid bacteria (LAB) are microorganisms that beneficial to the body that produce compounds which can kill pathogenic bacteria (Klaenhammer *et al*., 2005; Jansson, 2005). In addition LAB is also known as probiotics, antitumor, reduction in serum cholesterol and lipid, immunomodulators. (Ikeda *et al*., 2007). There are about 13 genera of bacteria that include as LAB are *Bifidobacterium, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weissella* (Salminen and Wright, 2004). LAB can be isolated from the gastrointestinal tract of broiler chickens that produce the genus *Lactobacillus sp*. (Sari *et al*., 2013; Mujnisa 2013), peking duck (Sumarsih *et al.,* 2014). Lactic acid bacteria can also be isolated from cow's rumen fluid that gets *Lactococcus lactis* spp. *lactis* and *Lactobacillus brevis*, (Suardana *et al.*, 2007). Lactic acid bacteria can also be obtained from the gastrointestinal tract orangutan.

Orangutan is one primate that is currently the most endangered in the world (Ginting, 2006). Results of studies of Bornean orangutan (*Pongo pygmaeus*) in Taman Safari Indonesia found the number of LAB 2.2 x 107 cfu/ml (Prasthani, 2012). In the Sumatran orangutan (*Pongo abelii*) there are 10^4 cfu/ml colonies LAB with discrete colonies beige, round, elevation umbonate and a Gram - positive bacteria (Putra, 2015). However LAB on the Sumatran orangutan not yet to be identified. The aims of this study to characterize the 16S rRNA gene of lactic acid bacteria (LAB) from the Sumatran orangutan (*Pongo abelii*).

Materials and Methods

Isolation Lactic Acid Acid Bacteria

The sample in this study is isolat lactic acid bacteria from sumatran orangutan (*Pongo abelii)* from Bukittinggi West Sumatera zoo.

Isolation Total DNA

Isolation LAB performed with standard cultivation methods by Oxoid (2015). Followed by isolation of LAB on Nutriet media Broth (NB) onwards gram staining and DNA isolation using Presto TM Total gDNA Mini Kit Bacteria.

Amplification of 16srRNA gene Fragment

PCR were peformed by using taq polymerase according to instrctions provided by manufacturer. A touchdown PCR program was implemented as follows: an initial denaturation step at 94^0C for 5 min, followed by 30 cyles, where denaturation was performed at 94^0C for 1 min, the annealing temparature was peformed at 72^0C for 2 min. In the first 10 cycles, the annealing temperature was continually decreased by 1^0C every cycle from 53 to 43^0C. The final extention was performed 10 min at 72^0C.

PCR product was determined by electrophoresis analysis, through 1.5% agarose and 1xTAE buffer (40mM Tris HCl, 40 mM acetate, 1.0 mMEDTA) under UV light.

Determination of DNA Sequence

Determination of DNA sequence (sequencing) is performed through commercial services in Macrogen Inc., Korea. Determination process is using the Dye Terminator (3'-dyelabelled dideoxynucleotide triphosphate), which includes several phases: setup templates, sequencing reaction. PCR product purification, and electrophoresis with fluorescence scanning.

Analysis of DNA Sequence Homology

Samples with the DNA sequence similarity of DNA sequences that have been deposited in GenBank sequence alignment can be detected through DNA samples with the data in the NCBI nucleotide through *http://www.ncbi.nlm.nih.gov site*. Alignment is done by using N BLAST program (Altschul *et al*., 1997), here in after, about 100 DNA sequences that have the highest similarity were analyzed using Clustal W (Safika, 2013).

Results and Discussion

Results Isolation of Lactic Acid Bacteria

Bacteria samples were bred back to the media Nutrient Broth (NB). Incubated for 18-24 hours, colonies that grow Gram staining. Pure bred colonies that grow back in NB liquid medium at a temperature of 37^0C for 18-24 hours to ensure that the correct sample to be observed is shaped bacillus. Furthermore, the bacteria that grow centrifuged at a speed of 7000X g. The cell pellet was then used for DNA isolation.

Gram Staining

Gram staining results against bacterial colonies are creamy, basil and elevation convex shape, and showed a purple color. The purple color indicates that the bacteria is classified as Gram positive bacteria. These results can be seen in figure 1. Gram staining is performed to ensure that the correct bacterial isolation results are lactic acid bacteria. The composition of LAB cells were observed entirely composed chain and clustering. This is in accordance with the characteristics of LAB which is a Gram - positive bacteria.

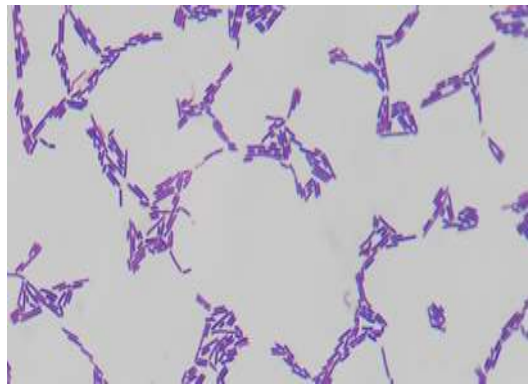

Figure 1. LAB origin sumatran orangutan shaped bacilli, Gram positive (purple) 1000x magnification

Gram positive bacteria are bacteria that can maintain gentian violet dye in the Gram staining process. The bacteria of this group will show blue-purple (violet)

whereas Gram-negative bacteria will be pink at the time of observation under a microscope. The color difference of Gram staining process is based on differences in cell wall structures that make up bacteria (Sulistyaningsih, 2008). Gram-positive bacteria cell wall consists of peptidoglycan layers which form a thick and rigid structure. This thick peptidoglycan layer makes the bacteria resistant Gram posisitf can flushing by alcohol, so as to prevent the escape of the main gentian violet dye. Most of the cell wall of Gram-negative bacteria such as lipids, so that when washing with alcohol lipid will be dropped and cause major gentian violet dye can not be maintained by the cell walls of bacteria on Gram stain process. (Smith and Husseys, 2005).

Lactic Acid Bacteria DNA Molecules

Total DNA Isolation is the first step to make the process of PCR. DNA isolation kit use Presto TM Mini gDNA isolation Bacteria kit. Lactic acid bacteria colony forming DNA extraction. To determine the DNA molecule electrophoresis agarose in 1x TBE buffer. Before electrophoresis of DNA suspension buffer mixed with colored cargo (loading dye). The addition of these colors serves to increase the density so that the DNA molecule is located at the bottom of the well, in addition to the dye is used to facilitate putting the DNA into the well and mark the DNA migration. Measures of success looks intact DNA isolation, not fragmented, and no tail, can be seen in Figure 2. According Safika *et al*., (2014), a good quality of DNA that are not degraded DNA patterns (smear) during visualization under UV light gel agarose. DNA so that it can be continued for amplification by PCR.

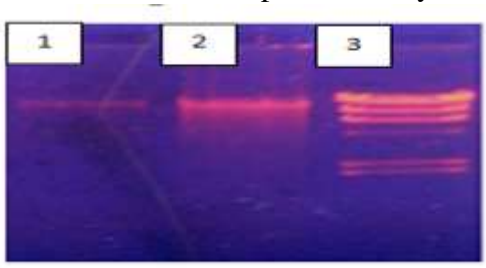

Figure 2. Results of electrophoresis of total DNA by using UV transluminator

Some electrophoresis buffer can be used for fractionation of nucleic acids such as, Tris-Acetate EDTA (TAE) or Tris-borate-EDTA (TBE). In general, the ideal buffer should generate less heat and good conductivity. For example, deviations from the optimal concentration of buffer (more concentrated) can generate enough heat to melt the gel (Boffey, 1984; Lodge *et al*., 2007).

Gene Amplification of 16S rRNA

The PCR process is highly dependent on the primers used. Primer is an oligonucleotide having 10 to 40 bp (bp = base pairs) and is complementary to the target DNA. The primaries that do not fit can lead to polymerization reactions between the target gene with primers (Kusuma, 2010). In this study, total genomic DNA was extracted from cultures of bacteria isolates the orangutan Sumatran primary use BacF 5 'AGA GTT TGA TCM TGG CTC AG3' used complements the area of sustainable on the domain of bacteria and other primary (UNIB) is (5 'GGT TAC STT GTT ACG ACT T 3 '), based on the area of sustainable universal 16S rRNA gene of *E. coli* that has a length amplicon/target DNA of about ± 1500 base pairs (bp). Both primer is general primer for bacteria (Aminin *et al.*, 2007).

In the PCR process also requires dNTPs (deoxynucleotide triphosphates) and a DNA polymerase enzyme (enzyme taq DNA polymerase). dNTPs are a mixture consisting of dATP (deoksiadenosin triphosphate), dTTP (deoxythymidine triphosphate), dCTP (deoksisitidin triphosphate) and dGTP (deoksiguanosin triphosphate). dNTP will be attached to the -OH group at the 3 'end of the primer to form a new strand that is complementary to the template DNA strand. DNA polymerase enzyme to function as catalysis for DNA polymerization reaction. These enzymes extend the DNA primer from the 3 'end (Handoyo and Rudiretna, 2001).

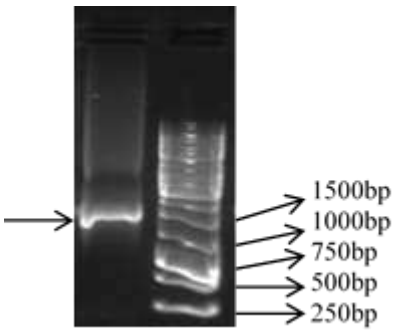

Figure 3. Results of agarose gel electropherogram 16S rRNA gene amplification LAB, ± 1500 bp amplicon length

Electrophoresis results of DNA amplicons have a size of ± 1500 bp (Figure 3). This proves that the composition of this process in the optimum condition that is in accordance with the long primary amplicons. In addition, the band also tailless amplicons, which means that the PCR process is going well. It can be concluded that the process of amplification of the 16S rRNA gene fragment LAB is successful.

DNA Sequence Homology Lactic Acid Bacteria

DNA sequencing is the process of determining the nucleotide sequence of the DNA amplicons results. The purpose of this process is to determine the type of nucleotide. Results of DNA sequence determination performed in Macrogen, *Inc., Korea* is shaped graph chromatograms. The result of the sequence and then analyzed peak electropherogram obtained by using DNA-Star software on Edit.seq program. The results of DNA analysis of nucleotides found in 1408 bp piece of 1500 bp nucleotide sequence of the nucleotide sequences were sequenced (Figure 4).

DNA sequencing or RNA-seq, allowing many advances in the characterization and quantification of the transcriptome. These developments include the initial mapping of transcription, strand-specific measurements, detection of gene fusion, and characterization of RNA. This development promises further advances in the application of quantitative sequencing of a very small number of isolates (Ozsolak and Patrice, 2011). Methodology for RNA-seq studies have provided more complete knowledge of the quantitative and qualitative aspects of the biology of transcription in prokaryotes and eukaryotes (Wang, *et al*., 2009). LAB nucleotide sequences that have been analyzed, then characterized back in NCBI through via the website *http://www.ncbi.nlm.nih.gov*. BlastN program (Basic Local Alignment Search Tool nucleotide). Characterization of these genes aims to determine the nucleotide sequence similarity or proximity of a sample with DNA sequences that have been deposited in the Gene Bank. In addition, about 100 DNA sequences that have the highest similarity with footage downloaded via the same site. Files are stored in the form FASTA (text) to facilitate editing. BlastN program is aimed at aligning methods of bioinformatics of genomic DNA sequences, rRNA and tRNA or mRNA containing a mixture of coding and noncoding sensitive. The analysis is done by comparing the incoming data with thousands of other data available in the database. A sequence of DNA sequencing results can be known resemblance to sequences stored in the database (Zhang, *et al*., 2000).

Figure 4. The results of the analysis of gene sequences LAB. The length of the analyzed 1408 bp bases

BlastN program parameters are similarities (Ident), query and the value of the E-value. The results showed that the samples BlastN LAB isolates have a percentage of similarity (Ident) 89% with *Lactobacillus helveticus* strain IMAU50151, the query 94% and the value of the E-value generated is zero. BlastN results can be seen in Figure 7. The Ident is the percentage of

matching nucleotides between nucleotide sequence with nucleotide sequence database query, where the results of this study, the similarity of 89%. Query shows how much amount of sample length nucleotide sequences that have been matched. Rated E value is an important parameter to consider in the determination of the proximity or similarity with the DNA that has been deposited in Genbank. Rated E value is the result of mathematical calculations and give the possibility to the benchmark results. In this analysis results obtained E- value generated value is zero, it indicates sequences homologous to sequences contained in the Genbank.

Figure 5. Results of gene sequences LAB BlastN Sumatran orangutan; 100 DNA sequences that have the highest similarity

Further characterization of the genes discovered through BlastN LAB orangutan has a gap of 5% with a bacterial gene *Lactabacillus helveticus* strain IMAU50151 (89% similarity). Visible identity of 1202 base pairs which have in common (89%) of 1356 base pairs were compared. With a gap of 5% or 70 base pairs from 1356 that can not be read (Figure 6).

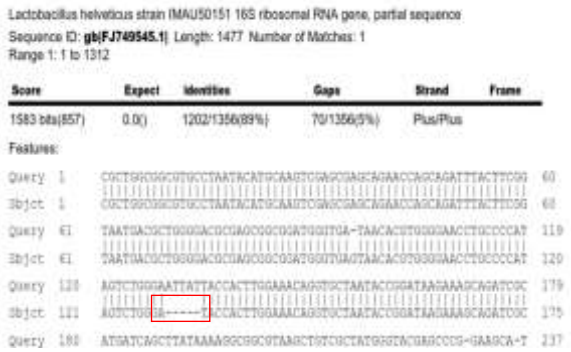

Figure 6. Gap Analysis LAB gene sequences of Sumatran orangutans *Lactabacillus helveticus* strain IMAU50151. [ ] Signs indicate Gap.

The alignment of sequences is the process of preparing/setting two or more sequences. Sequence alignment is a method in the analysis of sequences. This method is used to search for sequences similar or identical in sequence data bases, while also studying the evolution of sequences from the same ancestor (common ancestor). In alignment results found sign "|" which indicate a match or a match between the two sequences. Incompatibility (mismatch) in alignment associated with the mutations, while the gap (the gap) marked with "-" associated with the insertion or deletion (Mount, 2001).

DNA Sequence Alignment

Results of BlastN to isolate DNA samples Sumatran orangutan (*Pongo abelii*) are not the same, so the analysis is needed to keep the length and position of the same nucleotide bases. This analysis using ClustalW on Mega 6 (Tamura *et al*., 2011). When it is obtained in the form of a long and nucleotide positions are grouped into a single file as can be seen in Figure 7.

ClustalW is a bioinformatics program for alignment of multiple sequences at once (multiple alignment). From the results indicate results Clustal alignment (alignment) LAB isolate genes origin Sumatran orang utan (*Pongo abelii*) is *Lactobacillus helveticus* strain IMAU50151

with the similarity of 89%. Proximity to the *Lactobacillus helveticus* strain IMAU50151 of the results of this analysis together with the results of BlastN.

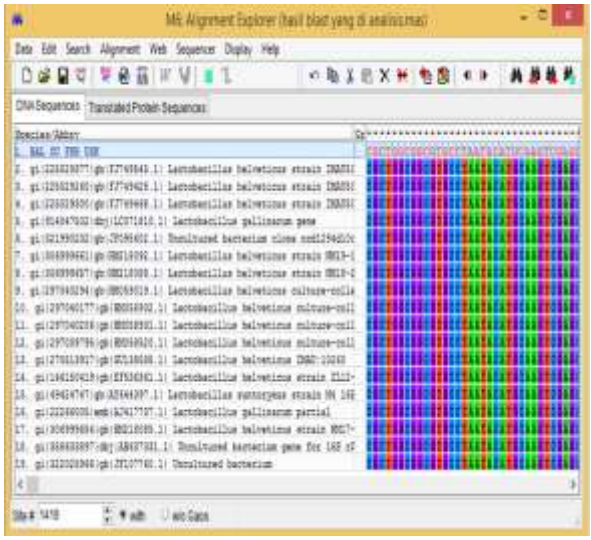

Figure 7. Results Gen alignment LAB sumatran orangutan with Clustal W Mega.6

According to Janda and Abbott (2007) if the percentage of homology have close to 100% or above 97% can be confirmed as a species but otherwise if homology is less than 97% probability of these isolates was a new species or a species yet to be confirmed. Meanwhile, according to the data in Wheeler and Bhagwat (2007) if the identity max value of 99% indicates that the isolates are considered the same species. While the value of ≥ 97% can be stated that isolates compared are in the same genus and if the homology between 89-93% show different family. So that the results of this study Sumatran orangutan gene is new and different kind of species and genus Lactobacillus or the genus/species which has not been reported in GenBank. Further analysis is required so that phylogenetic analysis to look at the evolutionary relationships of genes LAB sumatran orangutan and to determine the branching formed by isolates through observation of the position occupied among other species or species comparison.

Probiotics of the genus Lactobacilli now increasingly known for its involvement in the commercial healthcare functions that are often isolated from the intestinal microbes, such as *Lactobacillus acidophilus, L. rhamnosus, L. johnsonii, L. paracasei, and L. reuteri* has a lot of benefits to the host because of its influence the physiological processes of the intestine (Rong *et al*., 2015). Characteristics of probiotics was associated with the presence of membrane molecules and structures on certain bacteria like peptidoglycan, teichoic acid, polysaccharides and the protein surface that serves to evoke a different response (Leeber *et al*., 2008).

Lactobacillus helveticus is widely used in the manufacture of Swiss cheese and Italian cheese and fermented beverages (Fortina, *et al*., 1998). Lactobacillus helveticus known to have protease enzyme activity (Savijoki *et al*., 2006). Proteolytic bacteria function relating to the ability to reduce the bitter taste and speed up the process of making cheese, as well as for the release of bioactive peptides in milk-based food products. (Ardo and Pettersson, 1988; Drake *et al*., 1996).

L. helveticus known as LAB produce probiotics because of its ability to survive in the gastrointestinal tract has many features commonly known as probiotics, such as the ability to survive in the gastrointestinal tract, attaches to epithelial cells, and inhibit bacterial pathogens. *L. helveticus* also able to prevent gastrointestinal infections, and affects the intestinal microbial composition (Slattery *et al*., 2010). Another benefit of the bacterium L. Helveticus to humans may be enhancement of bioavailability of nutrients and elimination of allergen molecules derived from food (Taverniti and Guglielmetti, 2013). The results of the study (Rong *et al*., 2015) Lactobacillus helveticus strain probiotic properties and NS8 showed good immunomodulatory capabilities, with the potential for development of commercial food and therapies for inflammatory diseases.

While *Lactabacillus helveticus* strain IMAU50151 is a lactic acid bacteria found in goat milk cake in Yunan China (Bao *et al*., 2011).

Conclusion

Based on these results it can be concluded that the lactic acid bacteria (LAB) from the sumatran orangutan (*Pongo abelii*) has a closeness with lactic acid

bacteria strain of *Lactobacillus helveticus* strain IMAU50151 and Gen LAB sumatran orangutan (*Pongo abelii*) has proximity by 89% with *Lactobacillus helveticus*. A possibility that these bacteria is a species/genus is new or has not been reported in Genbank.

Suggestion

Need to do more analysis of the evolutionary relationships of LAB origin Sumatran orangutan (*Pongo abelii*) to point of constriction of the phylogenetic tree. It is also necessary to analyze the lactic acid produced.

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